Characterization of the annular lipid shell of the Sec translocon

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A R T I C L E   I N F O

Article history:
Received 12 May 2015
Received in revised form 24 June 2015
Accepted 26 June 2015
Available online 27 June 2015

Keywords:
Sec translocon
Styrene-maleic acid
SMALP
Translocation
Lipid–protein interaction

A B S T R A C T

The bacterial Sec translocase in its minimal form consists of a membrane-embedded protein-conducting pore SecYEG that interacts with the motor protein SecA to mediate the translocation of secretory proteins. In addition, the SecYEG translocon interacts with the accessory SecDFyajC membrane complex and the membrane protein insertase YidC. To examine the composition of the native lipid environment in the vicinity of the SecYEG complex and its impact on translocation activity, styrene-maleic acid lipid particles (SMALPs) were used to extract SecYEG with its lipid environment directly from native Escherichia coli membranes without the use of detergents. This allowed the co-extraction of SecYEG in complex with SecA, but not with SecDFyajC or YidC. Lipid analysis of the SecYEG-SMALPs revealed an enrichment of negatively charged lipids in the vicinity of SecYEG, which in detergent assisted reconstitution of the Sec translocase are crucial for the translocation activity. Such lipid enrichment was not found with separately extracted SecDFyajC or YidC, which demonstrates a specific interaction between SecYEG and negatively charged lipids.

1. Introduction

The Sec translocase is conserved and essential in all kingdoms of life [1]. In bacteria, the Sec translocase mediates the translocation of secretory proteins across and the integration of membrane proteins into the cytoplasmic membrane. For secretion, the translocase consists of the protein conducting pore SecYEG and the associated soluble ATP-driven motor protein SecA [2,3]. In addition, SecYEG also interacts with other membrane proteins such as SecDFyajC [4] and YidC [5]. SecDFyajC supports proton motive force driven protein translocation [6], while YidC facilitates membrane protein insertion either by itself [7] or in cooperation with the SecYEG translocon [5,7]. Protein translocation is a dynamic process in which cycles of ATP binding and hydrolysis by the SecA motor results in the stepwise translocation of unfolded preproteins through the SecYEG pore. Anionic lipids play an important role in this process, and are needed to activate the SecA motor and essential for protein translocation [8]. Most studies on the role of phospholipids in translocation have been performed in vitro using detergent-based purification and protein reconstitution into (proteo-)liposomes [9,10] or into lipid nanodiscs that are stabilized by a membrane scaffold protein (MSP) [11]. However, little is known about the native lipid environment in the vicinity of the Sec translocase. Previously, purified and detergent extracted SecYEG complex was shown to contain cardiolipin [12], suggesting that this lipid is tightly bound. Detergent-based purification methods, however, may not provide an accurate estimate on the bound lipids as certain lipid species may be more readily lost during the detergent solubilization than others [13,14].

Styrene-maleic acid lipid particles (SMALPs), also termed native nanodiscs, are a new tool to extract membrane proteins together with their lipid environment [15–18] (Fig. 1). In SMALP generation, styrene-maleic acid (SMA) copolymers (Fig. S1) integrate into lipid membranes and convert them into lipid nanodiscs of 10–12 nm in diameter as shown by structural analysis [19,20]. The SMA copolymers form a “bracelet” encircling the lipid membrane with the styrene moieties intercalated between the lipid acyl chains [20]. In this manner, a wide variety of lipids with different acyl-chain lengths and headgroups are trapped [21]. The SMALP method was previously used to purify membrane proteins from liposomes [15,16,22] but it can also be applied to study membrane proteins extracted from native membranes [18, 23–26]. In the latter case, the membrane proteins (complexes) are co-extracted with the native bound lipids, and through lipid analysis of the SMALPs, detailed information can be obtained about the composition of annular lipid shell. In addition, the method can be used to isolate stable membrane protein complexes from native membranes providing information on the interacting protein species as well as their stoichiometries.

Abbreviations: SMALP, styrene-maleic acid lipid particle; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; TLC, Thin Layer Chromatography; LC–MS, liquid chromatography–mass spectrometry; FRET, Förster resonance energy transfer.

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http://dx.doi.org/10.1016/j.bbamem.2015.06.024
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In this study we employ SMALP generation to characterize complex formation of the SecYEG translocon within its native environment. We extract SecYEG from *E. coli* membranes using SMA copolymers and identify interacting proteins that co-purify. Furthermore, we examine the lipid composition in the vicinity of the SecYEG complex and determine its impact on protein translocation.

2. Materials and methods

2.1. Chemical and reagents

The 3:1 pre-hydrolyzed styrene-maleic acid (SMA) copolymer was purchased from Malvern Cosmeceuticals. Lipids were purchased from Avanti Polar Lipids. The following lipids were used: *Escherichia coli* phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL); dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylglycerol (DOPG), and PE C24:0 (1,2-dilauroylphosphatidylethanolamine). HPLC-grade methanol, ethanol, isopropanol, chloroform, trimethylamine, acetonitrile, ammonium formate, formic acid, and molybdenum blue reagent were purchased from Sigma Aldrich. Ni-NTA agarose was from Qiagen and PVDF membrane was from Roche Diagnostics. Thin Layer Chromatography (TLC) silica gel plates were purchased from Merck Millipore and Shim-pack XR-ODS II column (75 mm × 3.0 mm inner diameter, particle size 2.2 μm) was from Shimadzu. Cy5-maleimide was purchased from GE Healthcare and Atto532-maleimide was purchased from Atto-Tec.

2.2. Membrane protein expression and purification

SecYEG with N-terminal His-tagged SecY [27] was over-expressed in *E. coli* SF100 [28]. Cells were harvested at 8,000 g for 15 min and resuspended in 50 mM Tris–HCl pH 8. Cells were lysed by French press treatment at 13,000 psi and cell debris was removed by centrifugation at 12,000 g for 15 min. The membrane fraction was collected with ultracentrifugation at 200,000 g for 30 min and resuspended in 50 mM Tris–HCl pH 8, 200 mM NaCl. A stock solution of 10% (w/v) SMA was prepared in 50 mM Tris–HCl pH 8. For extraction, the membrane containing SecYEG was incubated for 30 min at 25 °C in 50 mM Tris–HCl pH 8, 200 mM NaCl and 3% (w/v) SMA. The insoluble material was removed by centrifugation at 100,000 g for 30 min at 4 °C and the supernatant containing SecYEG was incubated overnight at 4 °C with Ni-NTA agarose. The slurry was added to the column and was washed five times with 10 bed volume of 50 mM Tris–HCl pH 8, 200 mM NaCl. Elution was performed by adding 1 bed volume of 50 mM Tris–HCl pH 8, 200 mM NaCl, 250 mM imidazole. *E. coli* membrane vesicles containing over-expressed levels of His-tagged YidC [29] or SecDFyajC with C-terminal His-tagged SecD [30] were isolated and subjected to SMA extraction as described above. The proteins were analyzed by SDS-PAGE and were also transferred to a PVDF membrane for western blot analysis.

2.3. Lipid analysis

Lipids were extracted from the samples according to the method of Bligh & Dyer [31] and solubilized in chloroform for Thin Layer Chromatography (TLC) analysis. The lipid extracts were spotted onto a TLC silica gel plate and *E. coli* lipids (PE, PG, and CL) were used as references. Lipid separation was performed with the mobile phase chloroform, ethanol, water and triethylamine in the ratio of 35:26:4:35 and were visualized by molybdenum blue [32]. Liquid chromatography–mass spectrometry (LC–MS) analysis were performed in an Accelea1250 high-performance liquid chromatography (HPLC) system coupled with electrospray ionization mass spectrometry (ESI–MS) Orbitrap Exact (Thermo Fisher Scientific). The samples were subjected to Bligh & Dyer extraction with the addition of PE C24:0 (C12:0/C12:0) as the internal standard and were solubilized in methanol. Five micro-liter of lipid extracts were injected to a Shim-Pack XR-ODS II column (75 mm × 3.0 mm inner diameter, particle size 2.2 μm) with a flow rate of 0.3 ml/min. LC separations were obtained using a gradient of mobile phase A consisted of 10 mM ammonium formate with 0.1% formic acid in water–acetonitrile (40:60, v/v), and mobile phase B consisted of 10 mM ammonium formate with 0.1% formic acid in acetonitrile–isopropanol (10:90, v/v) at 55 °C for 20 min as described [33]. The column effluent was injected directly into the Exactive ESI–MS Orbitrap operating in negative ion mode. The identification of lipid was performed using LIPID MAPS online searching tools (http://www.lipidmaps.org) [34] and data analysis was performed using the Thermo XCalibur processing software.

2.4. Formation of proteoliposomes

Synthetic phospholipids were mixed in the desired ratio in chloroform, dried under nitrogen, and incubated overnight under vacuum. The dried lipid films were resuspended in 10 mM Tris–HCl pH 8.0, 1 mM DTT to the final lipid concentration of 10 mg/ml and were dispersed using a bath sonicator. Reconstitution of detergent purified SecYEG [35] in liposomes of different lipid compositions were performed by the Bio-Beads method as described [36]. Liposomes were destabilized by 0.5% Triton X-100 and incubated with purified SecYEG. Detergent was removed overnight using Bio-Beads SM-2 adsorbents, and proteoliposomes were collected by ultracentrifugation and resuspended in 50 mM Tris–HCl pH 8, 50 mM KCl, 10% glycerol.
2.5. Membrane protein labeling

Whole cells over-expressing the SecYEG complex containing single cysteine residues at position 148 [37] were labeled with the fluorescent probe Cy5-maleimide. Cells were lysed by French press at 13,000 psi and the cell lysates were centrifuged at 12,000 g for 15 min to remove unbroken cells. The membrane fractions were collected with ultracentrifugation at 200,000 g for 30 min, extracted with SMA, and purified with Ni-NTA affinity chromatography. The preprotein proOmpA fused to dihydrofolate reductase (proOmpA-Dhfr) containing a unique cysteine residue at position 282 was labeled with the fluorescent probe Atto532-maleimide as described [38]. The purification and labeling efficiency were evaluated by SDS-PAGE and absorbance spectroscopy.

2.6. Protein translocation assays

The activity of the SecYEG proteoliposomes of different lipid compositions were tested by in vitro translocation assays using the fluorescently labeled proOmpA as described [39]. The translocation of proOmpA (50 nM) was performed in the translocation buffer (50 mM HEPES-KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml BSA, 10 mM DTT, 2 mM magnesium acetate, 10 mM phosphocreatine, 50 μg/ml creatine kinase) in the presence of SecYEG proteoliposomes (200 nM), the motor protein SecA (200 nM), and the chaperone SecB (400 nM). The translocation reaction was started by the addition of 2 mM ATP, incubated for 10 min at 37 °C, and was terminated by chilling the samples on ice following by proteinase K treatment. The translocated protease resistant proOmpA was precipitated with trichloroacetic acid (TCA) and analyzed by SDS-PAGE.

The activity of SecYEG-SMALP was assessed using a Förster resonance energy transfer (FRET) based assay on an Amino Bowman spectrofluorometer [37]. SMALPs containing SecYC148EG-Cy5 (100 nM) were incubated at 37 °C with pre-folded proOmpA-Dhfr-Atto532 (200 nM) and the motor protein SecA (1 μM) in the translocation buffer. Translocation reactions were initiated by adding 5 mM ATP or AMP-PNP. Donor fluorophore Atto532 was excited at 525 nm and FRET activity was measured as a change in the fluorescence acceptor emission at 670 nm.

3. Results

3.1. SecA co-purifies with SecYEG in SMALPs via protein–protein interaction

We applied the SMALP methodology for the extraction of SecYEG from native E. coli lipid membranes. Total membrane fractions containing over-expressed levels of histidine-tagged SecYEG were solubilized with SMA copolymers to purify SecYEG-SMALPs using Ni-NTA affinity chromatography. SDS-PAGE analysis revealed the typical bands corresponding to SecY (37 kDa) and SecEG (13–15 kDa) (Fig. 2A), demonstrating that the SMA copolymer is able to extract the SecYEG complex from the membrane. Interestingly, we also observed an additional upper band at 100 kDa in the purified sample. Western blot analysis revealed that the band corresponds to SecA, which is also present in the total membrane fraction (Fig. 2B). SecA is known to associate with SecYEG but this interaction is disrupted by detergent [2,3,40]. Apparently, with the SMALP technology, the interaction is, at least partially preserved.

Since SecYEG also functionally associates with SecDF [4] and YidC [5], we performed western blot analysis using anti-SecD, SecF, or YidC antibodies. If such complexes are stable during SMA extraction, at least a subfraction of the overexpressed SecYEG is expected to form a complex with these membrane proteins that are present at native levels. However, neither SecDF nor YidC was found to be present in the SecYEG-SMALPs as evidenced by immunodetection (Fig. 2B), whereas the indicated proteins were readily immunodetected in the membrane vesicles and the crude SMA extracted membrane protein fraction. To validate this finding, we performed SMA extraction and Ni-NTA purification of His-tagged SecDF and His-tagged YidC. SDS-PAGE and western blot analysis show that SecA and SecY do not co-purify with the SecDF-SMALPs (Fig. 2D) nor does SecA co-purify with the YidC-SMALPs (Fig. S2). SecY did also not co-purify with the YidC-SMALPs (not shown). This demonstrates that association of SecA with the SecEG-SMALPs is due to the specific binding to the SecYEG complex, and not the result of an interaction between SecA and the lipids present in the SMALPs. Interestingly, YidC co-purifies with SecDF, while SecY does not (Fig. 2D) indicating that YidC-SecDF complexes are more prevalent in the membrane than YidC-SecYEG complexes [5]. Thus, also high molecular weight membrane protein complexes (~160 kDa) can be purified in SMALPs that are otherwise separated during detergent-based purifications.

3.2. Negatively charged lipids are enriched in the vicinity SecYEG complex

The E. coli membrane consists of about 70% of phosphatidyethanolamine (PE) and 30% of negatively charged lipids, mainly phosphatidylglycerol (PG) and cardiolipin (CL) [41]. SMA-extraction of the membranes allows the determination of the lipid composition of the annular lipid shell surrounding membrane proteins. We performed thin-layer chromatography (TLC) to analyze the lipid composition on the purified SecYEG-SMALPs and compared this to the lipid extracts from total E. coli membranes and from the crude SMA-solubilized fraction. TLC analysis shows the presence of PE, PG, and CL in all samples (Fig. 3A) demonstrating that the lipids were indeed extracted from the membranes into SMALPs together with the SecYEG complex.

The TLC analysis also suggests an enrichment of PG and CL in the SecYEG SMALPs at the expense of PE. However, for quantitative analysis, we performed liquid chromatography--mass spectrometry (LC--MS). Lipids were first separated according to their acyl chains followed by mass determination and quantitation of the detected species. PE C12:0/C12:0 was used as the internal standard for the relative quantification of lipids within the samples. Lipid extracts from purified SMALPs, the SMA-extracted fraction, and total membranes were analyzed for the presence of the various lipid species. From ESI--MS spectra we identified lipid species in the samples with the mass range from m/z 600 to 800 for [M-H]-- ions of PE and PG, and m/z 1300 to 1500 for [M-H]-- ions of CL (Table S1 and Fig. S3). For example, the PE C32:1 (m/z 688:49) contains the total carbon number of 32 with a single unsaturation, most likely harboring C16:0 and C16:1 fatty acyl chains. As expected, PE was found to be the major lipid species in all samples with the most abundant PE species present at m/z 702:51 corresponding to PE C33:1 (Table S1 and Fig. S3). The detected lipid species were quantified and compared between the samples. Our data show that the composition of the total PE, PG, and CL species in the SMA-extracted fraction is not significantly different from that of the total membrane (Fig. 3B), confirming earlier reports [18,21] that SMA does not preferentially solubilize specific lipid species. Interestingly, an enrichment of negatively charged lipids was observed in SecYEG-SMALPs with an increase of ~40% in PG and ~90% in CL compared to the total membrane fraction (Fig. 3B). In contrast, lipid analysis of the SecDF-SMALPs (Fig. 4) and YidC-SMALPs (Fig. S2) did not show an increase of PG and CL relative to the total membrane fraction. These results indicate that negatively charged lipids specifically associate with the SecYEG complex. Additionally, there is no apparent preference for any specific lipid species with different acyl chain length in SecYEG SMALPs compared to the total membrane fraction (Fig. S4), further suggesting that the interaction relates to polar head group specificity.

3.3. Phosphatidylglycerol and cardiolipin are important for SecYEG activity

Previous studies showed that the presence of negatively charged lipid is important for protein translocation [8,10,12]. In order to confirm the dependency of protein translocation on negatively charged lipids,
we reconstituted SecYEG in liposomes with and without PG and CL. The activity of reconstituted SecYEG was tested by measuring the translocation efficiency of fluorescently labeled preprotein proOmpA into the proteoliposomes. The SecYEG concentration reconstituted in each batch of liposomes was adjusted to the same level prior to the translocation assays (Fig. 5, upper panel). The same DOPE concentration (70%) was used in all samples to mimic the PE concentration in the E. coli membrane. SecYEG showed translocation activity when reconstituted into DOPE:DOPG:CL (70:20:10, mol% phosphate) but was completely inactive when reconstituted into DOPE:DOPC (70:30,
prior to the translocation assays. The translocation levels are 12% of the input proOmpA.

The effect of negatively charged lipids on the SecYEG activity. SecYEG was
reconstituted into liposomes containing DOPE:DOPG (70:30, mol% phosphate) or DOPE:CL (70:30, mol% phosphate). Similar translocation activity was observed for SecYEG in those two liposomes (Fig. 5) demonstrating that the dependency is fulfilled by the presence of negatively charged lipids, and that there is no preference for either PG or CL. It should be stressed that DOPC is invariant in this assay as previously we have shown that the SecYEG complex is active in membranes containing DOPC such as for instance DOPE:DOPC:DOPG (30:40:30, mol% phosphate) [10].

Next, to find out whether the SMA extraction method can maintain the functional properties of the SecYEG translocon, we performed an in vitro translocation assay using Förster resonance energy transfer (FRET) as described previously for proteoliposomes [37]. The principle of this assay is based on the energy transfer between fluorophores conjugated to the SecYEG translocon (fluorescence acceptor) and the substrate preprotein (fluorescence donor) that occurs upon the formation of a translocation intermediate [37]. The fusion protein proOmpA-DhfR was used as a translocation substrate as it consists of the unfolded proOmpA precursor protein that is translocated and the dihydrofolate reductase (DhfR) domain that is tightly folded in presence of its ligands NADPH and MTX [42] (Fig. 6). Formation of a translocation intermediate occurs when the proOmpA part is translocated and the folded DhfR domain blocks further translocation. Establishment of this locked state is monitored in time as an increased emission of the fluorescence acceptor at the exit of the SecYEG pore due to the energy transfer from fluorescence donor on proOmpA-DhfR. FRET data show an increase in the acceptor fluorescence emission in presence of SecA and ATP but not with the non-hydrolysable ATP-analog AMP-PNP indicating the formation of a translocation intermediate in presence of ATP (Fig. 6). These results demonstrate that SecYEG-SMALPs are active in the translocation of preproteins.

Fig. 5. The effect of negatively charged lipids on the SecYEG activity. SecYEG was reconstituted in liposomes composed of the indicated synthetic phospholipids. The activity was tested by measuring the translocation of fluorescently labeled preprotein proOmpA into proteoliposomes. SecYEG concentration was adjusted to the same level prior to the translocation assays. The translocation levels are 12% of the input proOmpA.

Fig. 4. Lipid analysis of SecDF-SMALPs. A) TLC analysis on phospholipids extracted from total membranes (lane 1), SMA-extracted membrane protein fraction (lane 2), and SecDF-SMALPs (lane 3); B) LC–MS analysis of lipids in total membranes (black), SMA-extracted membrane protein fraction (light gray), and SecDF-SMALPs (dark gray). Error bars represent the standard deviation of two experiments.

4. Discussion

In this study, we report the application of SMALPs for the characterization of the bacterial Sec translocon. We successfully extracted and purified the SecYEG complex from native E. coli lipid membranes confirming that the SMA-extraction is a powerful detergent-free method for membrane protein isolation. Interestingly, we also observed that the soluble motor protein SecA co-purifies with SecYEG in the SMALPs demonstrating that native protein–protein interactions remain intact during SMA-extraction. This is a major advantage over detergent-based purification that disrupts protein interactions such as the SecA-SecYEG complex. In the cell, SecA exists in a soluble cytosolic form [43] and membrane bound forms, i.e., associated with the SecYEG complex [2,3,40] and with phospholipids [44–46]. When we apply the SMA purification method to SecDFyajC or YidC, no SecA co-purification was observed validating the specificity of the SecA-pull-down with the SecYEG-SMALPs. Importantly, this demonstrates that the bound SecA is not merely co-purified with SecYEG through anionic lipid binding. We also observed the association between the membrane proteins SecDF and YidC in SMALPs consistent with a previous study [30], confirming that SMALPs can be applied for the extraction of large membrane protein complex as recently shown for AcrB (~360 kDa) [47]. SecDFyajC and YidC were suggested to closely associated with SecYEG to form a so-called holo-translocon complex [48]. However, we did not observe the extraction of a SecYEG–SecDF or SecYEG–YidC complex, suggesting that the holo-translocon complex is either a labile structure that is not stable during SMALP extraction or the complex is present only transiently and not captured during our assay conditions.

The interaction of membrane proteins with lipids is important for their structural integrity and functionality [8,49]. An advantage of using SMA-extraction lies in the maintenance of native lipid–protein interactions allowing the determination of the lipid composition of the annular shell as recently shown for the SMA extracted KcsA channel [18]. Lipid analysis on SecYEG-SMALPs showed an enrichment of negatively charged lipids at the SecYEG complex. With the individual

mol% phosphate) (Fig. 5). These results confirm that the negatively charged lipids are indeed essential for protein translocation. To compare the effect of PG and CL on protein translocation, SecYEG was reconstituted into liposomes containing DOPE:DOPG (70:30, mol% phosphate) or DOPE:CL (70:30, mol% phosphate). Similar translocation activity was observed for SecYEG in those two liposomes (Fig. 5) demonstrating that the dependency is fulfilled by the presence of negatively charged lipids, and that there is no preference for either PG or CL. It should be stressed that DOPC is invariant in this assay as previously we have shown that the SecYEG complex is active in membranes containing DOPC such as for instance DOPE:DOPC:DOPG (30:40:30, mol% phosphate) [10].

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SecDF-SMALPs and YidC-SMALPs, a lipid composition similar to total *E. coli* membrane extract was observed, indicating a specific enrichment of anionic phospholipids at SecYEG. The enrichment of anionic lipids at SecYEG is in line with the crucial role of these lipids in protein translocation [8,10,46,50,51]. This role could be manifold. The motor protein SecA binds to anionic lipids [52,53], and requires these lipids for its role being essential for the dimerization of the SecYEG complex [12]. We further investigated the influence of negatively charged lipids on the SecYEG complex by measuring its translocation activity in the presence or absence of PG and CL, respectively. SecYEG is completely inactive in absence of negatively charged lipids confirming earlier observations [10]. By replacing the DOPC fraction in the DOPE/DOPC lipid mixture by either PG or CL the translocation activity in SecYEG proteoliposomes can be fully restored. This shows that neither PG nor CL is specifically required. In this respect, a recent study that investigated the physiological role of CL in *E. coli* showed that the inactivation of the three paralogous cardiolipin synthetase genes [54] results in a complete loss of CL biosynthesis but at the same time, this only marginally affected cell viability. Taken together, this demonstrates that there is no strict requirement for CL in protein translocation.

In summary, our study demonstrates the application of SMA-extraction for the characterization of the SecYEG translocon, its interaction with lipids and partner proteins. The lipid analysis indicates a preferential interaction of SecYEG with negatively charged lipids which are essential for the translocation activity. SMA-extraction allowed detergent free purification of SecYEG with the associated SecA motor protein including the surrounding lipid environment.

**Author contributions**

IP designed and performed the experiments, analyzed the data and wrote the paper; IK designed the experiments, supervised the work and wrote the paper; AC designed and performed the experiments; AD designed the experiments, supervised the work and wrote the paper.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgments**

This work was supported by Earth and Life Sciences (ALW) which is supported by the Netherlands Organization for Scientific Research (NWO) grant no 854.11.001. The work of I.P. was supported by a Bernoulli grant of the University of Groningen. The work of A.C. was supported by the research program of the biobased ecologically balanced sustainable industrial chemistry (BE-BASIC).

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamer.2015.06.024.

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